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Title of Dissertation: "Regulation of Brain Glucose Metabolic Patterns by Protein Phosphorlyation and Drug Therapy"

Name of Candidate: Nader Halim
Doctor of Philosophy Degree
30 March 2007

Dissertation and Abstract Approved:

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FINAL EXAMINATION FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

Name of Student: Nader Halim

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Time: 1:00

Place: Lecture Room A

DECISION OF EXAMINATION COMMITTEE MEMBERS:

	PASS	FAIL
Gabriela Dveksler, Ph.D. Department of Pathology Chairperson	✓	
Ajay Verma, M.D., Ph.D. Department of Neurology Major Advisor	✓	
Michael Schell, Ph.D. Department of Pharmacology Member	✓	
Stephen Davies, Ph.D. Department of Microbiology & Immunology Member	✓	
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Abstract

Title: REGULATION OF BRAIN GLUCOSE METABOLIC PATTERNS BY PROTEIN PHOSPHORYLATION AND DRUG THERAPY

Author: Nader Halim Ph.D. (2007)

Thesis Directed By: Ajay Verma M.D., Ph.D. Professor, Department of Neurology

ABSTRACT: Glucose metabolism is the main energy-producing pathway of the central nervous system under normal conditions. Thus the regulation of brain glucose metabolism is essential to maintaining homeostasis. This study aims to determine whether the phosphorylation status of the pyruvate dehydrogenase complex differentiates the metabolic phenotype of astrocytes and neurons *in vitro*; and to determine whether antipsychotic drug administration affects glucose metabolites *in vivo*. The results of this study demonstrate that the phosphorylation status of the pyruvate dehydrogenase complex underlies the glycolytic phenotype of astrocytes and the oxidative phenotype of neurons in culture. In cultured astrocytes this phenotype can be altered to resemble that of neurons by treatment with the pyruvate dehydrogenase activating compound dichloroacetate. These data also suggest a possible molecular mechanism for the metabolic exchange of monocarboxylates between astrocytes and neurons *in vivo*. We also demonstrate in rats that chronic antipsychotic administration results in an increase in postmortem lactate levels. We also show an increase in the postmortem lactate levels in the cerebellum of patients with schizophrenia. These results suggest that postmortem metabolic alterations in the brains of patients with schizophrenia may be the result of antipsychotic treatment rather than a primary feature of the disease.

REGULATION OF BRAIN GLUCOSE METABOLIC PATTERNS BY PROTEIN PHOSPHORYLATION AND DRUG THERAPY

by

Nader Halim

Thesis/dissertation submitted to the faculty of the
Molecular and Cell Biology Program
Uniformed Services University of the Health Sciences
In Partial fulfillment of the requirements for the degree of
Doctor of Philosophy 2007

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This work is dedicated to my Mother.

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Chapter I

Glucose metabolism is the major source of energy production in most organisms. Adenosine triphosphate (ATP) is the currency of energy used by all cells for synthetic and energy consuming processes. ATP generation is thought to have evolved in three stages. The first of which was anaerobic glycolosis, followed by photosynthesis, then mitochondrial oxidative metabolism. These three pathways either consume (oxidative metabolism or glycolosis) or produce glucose (photosynthesis). Almost all cells can utilize glucose for energy.

Overview of Glucose Metabolism

Once transported into (or produced in) the cell glucose is rapidly phosphorlyated to glucose 6-phosphate (G6P). G6P has three predominant fates (Fig. 1). It can be polymerized and stored in the form of glycogen or metabolized by either the pentose phosphate pathway or via glycolysis. In most tissues 80-90% of glucose oxidation occurs via glycolysis while the remaining 10-20% enters the pentose phosphate pathway. The primary function of the pentose pathway is the production of either nicotinamide adenine dinucleotide phosphate (NADPH) or ribose. Both pathways are anaerobic, yet unlike glycolysis the pentose pathway shunt does not consume or produce ATP. Glycolysis is the central pathway for carbohydrate metabolism. This pathway consists of ten reactions that convert one molecule of glucose to two molecules of pyruvate, generate two molecules of ATP, and reduce two molecules of nicotinamide adenine dinucleotide (NADH). Glycolytic enzymes are some of the oldest and most conserved enzymes on

earth. As photosynthetic organism changed the earth's atmosphere with the introduction of oxygen, oxidative metabolism surfaced.

With oxygen came the evolution of oxidative metabolism, which enabled the complete oxidation of glucose. The combination of these two metabolic arms of glucose oxidation resulted in the high yield ATP needed by complex organisms. Ultimately these two pathways converge on the pyruvate dehydrogenase complex (PDC).

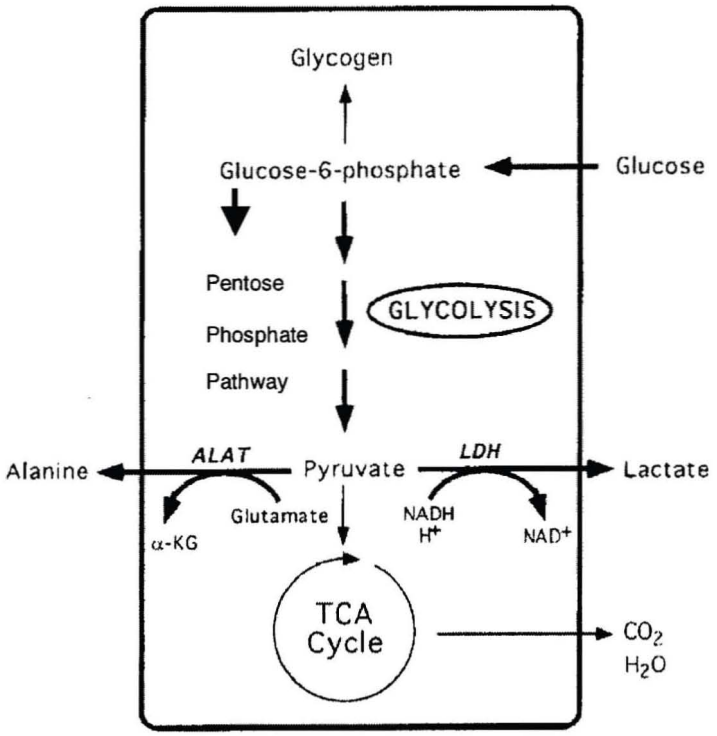

Figure 1. Simplified diagram of the metabolism of glucose with emphasis of the fates of glucose 6-phosphate and pyruvate. Modified from Tsacopoulos and Magistretti 1996.

Pyruvate (Monocarboxylate) Transporters

Prior to the continued oxidation of pyruvate via the PDC in the mitochondria, pyruvate must first be transported into the mitochondria. Over 30 years ago work from

the laboratory of Dr. Halestrap demonstrated the existence of two distinct lactate/pyruvate transporters (Halestrap 1975; Halestrap 1976). One of these transporters was found in the plasma membrane while the other was found on the mitochondrial membrane. The plasma membrane transporters were classified as moncarboxylate transporters (MCT). MCTs are a family of proton-linked transporters, individual MCT has slightly different substrate affinities as well as inhibitor specificities (Halestrap and Price 1999). The identification of the mitochondrial pyruvate transport has been harder to elucidate than the plasma membrane MCTs. To date the identity of the mammalian mitochondrial pyruvate transporters is unknown, though the yeast transporter was recently identified in the laboratory of Dr. Halestrap (Hildyard and Halestrap 2003). A mutant strain of yeast was identified which lack a single gene (YIL006w). △YIL006w yeast mitochondria lack the 41.9 kDa protein gene product and are unable to transport mitochondria. This was the first identified mitochondrial pyruvate transporter. The YIL006w gene products has little homology to any known mammalian protein, the most closely related protein have approximately 30% sequence identity though they do resemble three known mitochondrial genes (the mitochondrial folate transporter and two unknown mitochondrial genes with no known function (MGC4399 and NP_060625).

The Pyruvate Dehydrogenase Complex

The pyruvate produced from glycolysis has two possible fates depending on the organism, cell, and/or oxygen availability. In lower organisms and some cells of higher organisms, pyruvate is reduced in the process of fermentation to lactate, ethanol, or other fermentation products. Alternatively, pyruvate can be further oxidized to CO_2 and H_2O

via the citric acid cycle, which produces reducing equivalents for oxidation by the mitochondrial electron transport chain, resulting in high ATP production. Once transported into the mitochondria, pyruvate is oxidized by the multienzyme pyruvate dehydrogenase complex to yield NADH, acetyl CoA (ACoA), and CO_2. The reaction catalyzed by the PDC is irreversible and is the rate-limiting step of the citric acid cycle.

Structure of Pyruvate Dehydrogenase Complex

The irreversible oxidative decarboxylation activity of the PDC occurs via three sequential enzyme reactions that occur in a multienzyme substrate channel complex (Berg, Tymoczko et al. 2002). The PDC complex is composed of four subunits encoded by four gene products on different chromosomes. The first enzyme (E1) of the PDC is pyruvate dehydrogenase (PDH). This enzyme catalyzes the oxidation of pyruvate to CO_2 and H_2O. PDH is composed of two subunits E1α and E1β. The PDHA1 gene on the X chromosome encodes the E1α subunit, while the PDHB gene on chromosome 3p encodes the E1β subunit. The second enzyme (E2) of the PDC dihydrolipoyl transacetylase catalyzes the formation of ACoA. The final enzyme (E3) of the complex dihydrolipoyl dehydrogenase results in the generation of NADH. The four gene products that compose the PDC are encoded on nuclear DNA imported into the mitochondria and assembled into the complete multienzyme complex.

Regulation of the Pyruvate Dehydrogenase Complex

As the PDC occupies a central position in carbohydrate metabolism, its strict regulation is crucial to energy homeostasis. Activity of the PDC is regulated by both

covalent modifications and allosteric inhibition (Holness and Sugden 2003). Regulation of the PDC occurs primarily via phosphorylation of three serine residues on the E1α subunit (Korotchkina and Patel 1995; Korotchkina and Patel 2001; Korotchkina and Patel 2001; Holness and Sugden 2003; Korotchkina, Ciszak et al. 2004). Phosphorylation of E1α renders the enzyme complex inactive. Phosphorlyation of just one of the three serine residues (S293, site 1) renders the complex inactive, thus phosphorylation of this subunit reflects the activity of this reaction and subsequent reactions of the complex (Korotchkina and Patel 2001; Korotchkina and Patel 2001; Korotchkina, Ciszak et al. 2004). The phosphorylation and dephosphorylation of PDH occurs via four PDH specific kinases, pyruvate dehydrogenase kinases (PDK1-4) (Popov, Hawes et al. 1997; Bowker-Kinley, Davis et al. 1998) and two pyruvate dehydrogenase phosphatases (PDP1-2) (Popov, Kedishvili et al. 1993; Popov, Kedishvili et al. 1994; Gudi, Bowker-Kinley et al. 1995; Rowles, Scherer et al. 1996; Huang, Gudi et al. 1998).

The PDK isozymes are encoded by different genes and have tissue/cell specific distributions (Bowker-Kinley, Davis et al. 1998). All isozymes have the ability to phosphorlyate all three sites on the E1α subunit. However differences in isozyme expression, responsiveness, and biochemistry have been observed. PDK1 expression appears to have a limited tissue distribution and inducible under hypoxic conditions (Papandreou, Cairns et al. 2006). PDK2 is widely distributed in all mammalian tissues and is thought to be the ubiquitous kinase expressed in cells (Bowker-Kinley, Davis et al. 1998). PDK3 expression has a limited distribution while PDK4 expression is inducible during starvation (Wu, Sato et al. 1998). Two specific PDC phosphatases (PDP) are known (Huang, Gudi et al. 1998). Similar to the four PDK isozymes, both isozymes of

PDP have the ability to dephosphorlyate the three regulatory sites of Ê1α (Korotchkina and Patel 2001). Both isozymes PDP1 and PDP2 are Mg^{2+} dependent, though only PDP1 is Ca^{2+} sensitive (Huang, Gudi et al. 1998).

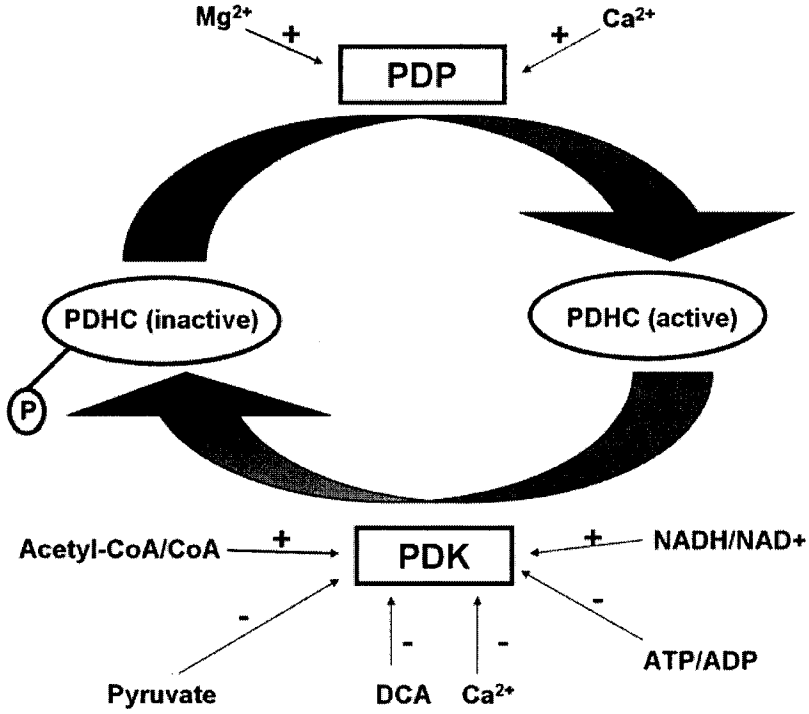

Figure 2. The regulation of the pyruvate dehydrogenase complex (PDHC) activity by phosphorylation and dephosphorylation by pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP).

Differential Metabolic Phenotypes of Cells

Metabolic pathways are not ubiquitously homogeneous in cells and tissues. The origins of this heterogeneity are diverse. Some cells have preferred metabolic pathways while other lack the capacity (enzymes or organelles) to perform certain pathways. The brain can utilize two sources of fuel for energy production: glucose and ketone bodies. Normally the brain functions exclusively on glucose oxidation (Clarke and Sokoloff

1999). Cardiac muscle can also utilize both ketone bodies and glucose yet cardiac muscle will utilize ketone bodies in preference to glucose (Berg, Tymoczko et al. 2002). Both cardiac muscle and brain contain the necessary enzymes to metabolize either glucose or ketone bodies. The enzymes involved in each metabolic pathway are differentially regulated in each cell type resulting in different metabolic phenotypes. Similar to brain, erythrocytes rely solely on glucose metabolism. Unlike brain cells erythrocytes lack mitochondria and only oxidize glucose to lactate. Similarly, active skeletal muscle does not oxidize glucose completely but oxidizes it to pyruvate and then releases it in its buffered form of lactate. Unlike erythrocytes skeletal muscle contains mitochondria. Initially this fermentation and resulting acidosis were thought to be a reflection of glycolysis out pacing oxidative metabolism, though recent evidence has begun to suggest that this view may be oversimplified. T-lymphocytes and astrocytes are highly glycolytic cells. Lymphocytes must be able to function in hypoxic regions, while the highly glycolytic metabolism of astrocytes may be crucial to neurons. These cells have mitochondria and so are presumably capable of metabolizing pyruvate. The fact that they largely produce lactate suggests that the mitochondrial metabolism of pyruvate may be tightly regulated in these cells.

Metabolic Exchange of Monocarboxylates Within and Between Cells

The segregation of metabolism into compartments reflects the evolutionary origins of these pathways. Within a cell, metabolic compartmentalization exists- glycolysis and the citric acid cycle occur in physically segregated regions of the cell.

Compartmentalization aids in the unification of the thousands of biochemical reactions within a cell.

The Cori cycle is the classical example of metabolic shuttling between skeletal muscle and the liver. In this cycle, active skeletal muscle oxidizes glucose predominantly to lactate where it enters circulation. Lactate is then transported into hepatocytes, converted to pyruvate via LDH, and then enters the gluconeogenic pathway where it is reconverted to glucose. The glucose produced is then released into systemic circulation for use by active skeletal muscle. Lactate may also be shuttled between cells in the brain.

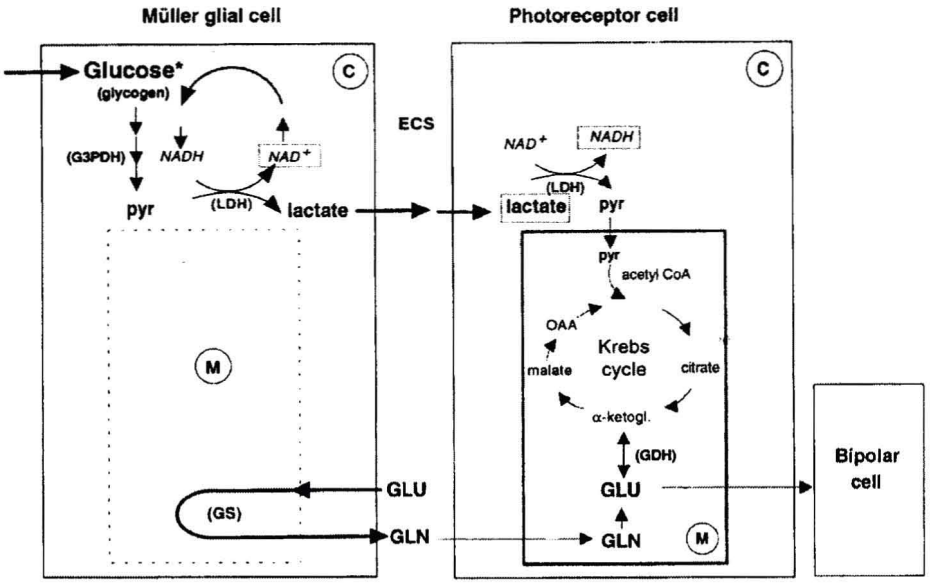

Figure 3. Diagrammatic representation of the metabolic stuttering of lactate between glia and photoreceptors in the retina of the guinea pig. Taken from Poitry-Yamate et al 1995.

Metabolite shuttling within the retina has been demonstrated in the honeybee as well as in the guinea pig. In this system alanine (in the insect) and lactate in the mammalian retina is produced in glia via the glycoltic pathway and shuttled to photoreceptor neurons

for use in mitochondrial oxidation (Figure 3). The metabolic shuttling of lactate has also been hypothesized o exist in the cerebrum. The astrocyte-neuron lactate shuttle hypothesis proposes that the metabolism of glucose in the brain occurs via metabolic shuttling between astrocytes and neurons (Pellerin and Magistretti 2003; Pellerin and Magistretti 2004; Pellerin 2005; Cerdan, Rodrigues et al. 2006). This hypothesis proposes that astrocytes uptake glucose and metabolize it to lactate. The resulting lactate is then imported into neurons where it is converted to pyruvate and subsequently oxidized. Metabolic coupling is thought be mediated by glutamatergic neurotransmission and regulated by differential expression of glucose transporters, lactate dehydrogenase isozymes, and monocarboxylate transporters isoforms (Leino, Gerhart et al. 1997; Laughton, Charnay et al. 2000; Debernardi, Pierre et al. 2003; Pierre and Pellerin 2005).

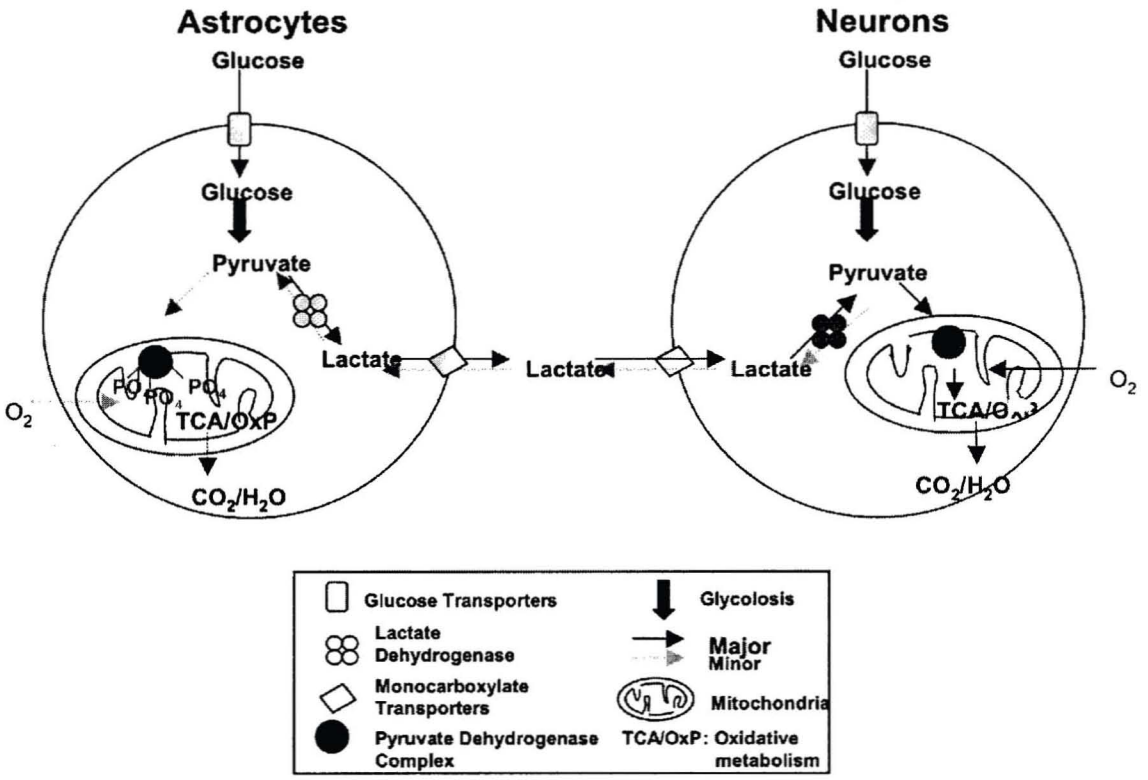

Figure 4. Diagram of the proposed molecular mechanisms of the metabolic shuttling of lactate between astrocytes and neurons.

Disorders of Monocarboxylate Metabolism

Deficiencies in the metabolism of the monocarboxylates pyruvate and lactate result in lactic acidosis. Genetic mutations in the PDH enzyme are a leading cause of congenital lactic acidosis and PDH abnormalities are associated with either metabolic or neurological syndromes. Primary metabolic deficiencies are evident shortly after birth and affected individuals present with lactic acidosis. The prognosis of a congenital primary metabolic deficiency is poor and often results in death. In contrast, neurological metabolic syndromes can present from shortly after birth into adulthood. Neurological

syndromes are associated with a milder phenotype that primarily affects the central nervous system with little or no somatic symptoms.

Most metabolic syndromes involve genetic mutations that result in a reduction of enzyme activity (Ho, Wexler et al. 1989; Matthews, Brown et al. 1994; Zeviani and Taroni 1994). Recent studies have suggested that alterations in the expression of numerous metabolic genes including subunits of the PDC may underlie the pathophysiology of schizophrenia (Prabakaran, Swatton et al. 2004). This notion is based evidence obtained from transcriptional (microarray), proteomic, and metabolomic data from the prefrontal cortex (PFC) and subsequently expanded to the liver and blood cells (Prabakaran, Swatton et al. 2004; Prabakaran, Wengenroth et al. 2007). The original study concluded that numerous metabolic alterations in the PFC result in increased anaerobic metabolism and a decrease in aerobic metabolism. These data led the authors to hypothesize that metabolic abnormalities in the PFC of patients with schizophrenia renders the PFC "vulnerable" to subsequent genetic or epigenetic insult and result in the precipitation of schizophrenia.

Schizophrenia: a Complex Disorder with an Unknown Etiology.

Schizophrenia is a complex neurodevelopmental disorder with an unknown etiology. Epidemiologic data indicates that this disorder has a prevalence rate of 0.5-1% regardless of geography, socioeconomic status, or ethnicity. Schizophrenia is a complex polygenic disorder with an epigenetic component (Weinberger 2002). Research into the etiology of schizophrenia is complicated by multiple factors including the lack of biomarkers, heterogeneity of the disease, substance misuse, and medication treatment

(Lipska, Deep-Soboslay et al. 2006). This is especially true of postmortem brain research since smoking, substance abuse, and antipsychotic medication induce numerous molecular alterations in the brain (Li, Konu et al. 2002; Law, Hutchinson et al. 2004; Abi-Dargham and Laruelle 2005; Van Gaal 2006). The most consistent findings in patients with schizophrenia are ventricular enlargement and deficits in working memory.

Pharamacological Clues to the Neurochemical basis of Psychosis

Clues to the neurochemical basis of psychosis can be inferred from the behavioral effects of several classes of drugs that effect neurotransmission. Dopamine agonists such as amphetamine and cocaine can induce symptoms analogous to psychosis, while DA antagonists are used to treat patients with schizophrenia and psychosis. Blockade of glutamatergic transmission with NMDA antagonists such as phencyclidne and ketamine and induces symptoms mimicking some aspects of schizophrenia. Finally serotonin agonists such as psilocybin and LSD can produce hallucinations thus these drugs also have the ability to mimic some aspects of schizophrenia.

Antipsychotics in the Treatment of Schizophrenia

In 1951-1952 Paraire and Sigwald made the first attempts to treat mental illness with the phenothiazine derivative chlorpromazine. They demonstrated that chlorpromazine was effective in reducing agitation as well as anxiety and held promise in treating patients with psychosis. In 1954 the use of chlorpromazine spread to North America and was used to treat a variety of conditions including schizophrenia as well as manic disorders. Chlorpromazine was found to be a potent antagonist of DA, specifically

acting to antagonize signaling at the D_2 receptor. Though D_2 receptor blockade is effective in managing psychotic symptoms it also produces a neuroleptic syndrome characterized by extrapyramidal neurological effects and an increase in the release of prolactin. Although chlorpromazine and haloperidol revolutionized the treatment of mental illness the sedating and neuroleptic side effects produced by "typical" antipsychotics have pronounced side effects. These adverse side effects were less pronounced in the next generation of antipsychotics developed. As these drugs lacked the "typical" neurolepic side effects they were are referred to as "atypical" antipsychotics. Pharmacologically the atypical antipsychotics are predominantly antagonists of DA signaling. Like typical anipsychotics they antagonize the D_2 receptor, but atypical antipsychotics also antagonize D_1, D_3, and D_4 receptors. Additionally unlike typicals, atypicals also modulate other neurotransmitter systems including the 5-HT_{2A}, 5-HT_{2C}, and H_1 receptors. Though atypicals lack the common neuroleptic side effects they have prominent side effects on metabolic function, often resulting in weight gain.

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Chapter 2 Significance and Summary

The proceeding chapter proposes that the phosphorylation status of the pyruvate dehydrogenase complex underlies the molecular basis of the differential metabolic phenotype of astrocytes and neurons observed *in vitro*. These data also suggest that the phosphorylation status of the pyruvate dehydrogenase complex may contribute to metabolic shuttling between astrocytes and neurons *in vivo*. We demonstrate that in vitro the expression and activity of lactate dehydrogenase is significantly higher in astrocytes than neurons. We show that the expression of the pyruvate dehydrogenase complex is higher in astrocytes while the activity of the complex is higher in neurons. Furthermore, we demonstrate the expression of the pyruvate dehydrogenase complex regulating kinases and phosphatases in both cell types though unique expression profiles were noted between astrocytes and neurons. Finally we show that the activity of the pyruvate dehydrogenase complex can be modulated by dichloroacetate, altering the glycolytic phenotype of astrocytes to a more oxidative phenotype resembling neurons.

This work adds additional evidence and a possible mechanism for the highly glycolytic phenotype of astrocytes. We also show data suggesting that neurons have an inherently oxidative metabolic phenotype.

Chapter 2: Phosphorylation Status of Pyruvate Dehydrogenase Distinguishes Metabolic Phenotypes of Rat Cerebral Cortical Astrocytes and Neurons

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Abbreviation: PDH, pyruvate dehydrogenase; DCA, dichloroactetate; LDH, lactate dehydrogenase; PDC, pyruvate dehydrogenase complex; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); pyruvate dehydrogenase kinase, PDK; pyruvate dehydrogenase phosphatase, PDP; MCT, monocarboxylate transporter

Abstract

Glucose metabolism in nervous tissue is thought to occur in a corporate manner with astrocytes contributing in large part to glycolytic metabolism and neurons being the primary site of glucose oxidation. Differential expression of glucose transporter isoforms, lactate dehydrogenase isozymes, and monocarboxylate transporters may explain the commitment of astrocytes towards producing large amounts of lactate from glucose. However, mammalian astrocytes and neurons both contain ample mitochondria, and it remains unclear why neurons oxidize glucose, lactate, and pyruvate to a much larger extent than astrocytes. It also remains unknown why some preparations of astrocytes are capable of oxidizing glucose completely. Here we report that both astrocytes and neurons express all components of the pyruvate dehydrogenase complex (PDC), the rate-limiting step for pyruvate entry into the Krebs cycle. However, PDC activity is kept strongly inhibited in astrocytes through phosphorylation of the pyruvate dehydrogenase α (PDHα) subunit while neuronal PDC operates close to maximal levels with much lower levels of phosphorlyated PDHα. Dephosphorylation of astrocytic PDHα restores PDC activity and lowers lactate production. Our findings suggest that the metabolic phenotype of astrocytes may be far more flexible that previously believed and that neurons may instead be more rigidly committed to glucose oxidation.

Introduction

The brain continuously consumes more glucose than any other mammalian organ (1, 2). However, complete cellular metabolism of glucose by brain tissue appears to occur in a corporate manner with distinct brain cell types performing the brunt of anaerobic vs. aerobic processing (3). Glycolysis, the cytosolic and anaerobic arm of glucose metabolism, has been proposed to take place largely in astrocytes while mitochondrial oxidation of the glycolytic endproducts pyruvate and lactate is believed to occur predominantly in neurons (4-6). This nervous tissue specific metabolic compartmentation, which has most clearly been established in insect and mammalian retina (7, 8), also requires the transfer of glycolytic endproducts from astrocytes to neurons. The astrocyte-neuron lactate shuttle hypothesis proposes that excitatory glutamatergic neurotransmission in the cerebral cortex enhances astrocytic glycolysis, which in turn generates lactate for transfer to and utilization by active neurons (9-11). Evidence supporting this hypothesis includes the unique anatomical relationship between brain capillaries, astrocytes and synapses, which favors a sequential transport and metabolism of glucose through these compartments. Brain glycogen is also found largely in astrocytes and can generate lactate for use by axons (12). Neurons on the other hand can survive in glucose-free media containing lactate or pyruvate (13) and *in vivo* hypoglycemic neuronal injury can be reversed with pyruvate infusion (14). Glutamate also stimulates glycolysis in astrocyte cultures but promotes glucose oxidation in neuronal cultures (15-17). These observations have led to a view of astrocytes largely being glycolytic cells with neurons displaying greater metabolic flexibility. This view has

been challenged by studies that suggest that astrocytes are highly capable of glucose oxidation (18).

The molecular mechanisms allowing segregation of glucose metabolic pathways between brain cell types have not yet been completely elucidated, but significant focus has been placed on lactate dehydrogenase (LDH) and monocarboxylate transporters (MCT). High rates of pyruvate conversion into lactate via LDH are necessary in cells that derive ATP largely from glycolysis, since this action maintains a high cytosolic NAD^+/NADH ratio and avoids an upstream block in glycolysis at glyceraldehyde-3-phosphate dehydrogenase (19). Astocytes express LDH isoforms that favor lactate formation, as well as MCT isoforms that favor lactate efflux (20-22). However, both neurons and astrocytes in the mammalian brain express ample mitochondria, and the molecular mechanisms that limit pyruvate oxidation by astrocyte mitochondria are not apparent. Cells in peripheral organs are well known to alter the routing of mitochondrial fuel oxidation depending upon physiological state and nutrient availability (23). A major molecular control point that allows liver and striated muscle cells to switch from mitochondrial oxidation of glycolytic endproducts to other fuels is the mitochondrial multienzyme pyruvate dehydrogenase complex (PDC). This large complex performs three reactions by three distinct components referred to as E1 (pyruvate dehydrogenase or PDH; composed of two subunits, PDHα and PDHβ), E2 (dihydrolipoyl acetyltransferase, DLAT) and E3 (dihydrolipoyl dehydrogenase, DLD), which together catalyze the irreversible oxidative decarboxylation of pyruvate to acteyl-CoA, CO_2, and NADH.

PDC activity can be dynamically regulated by the differential expression of its constituent proteins or by phosphorylation of the PDHα subunit (24-27). The control of PDHα phosphorylation is accomplished by a set of 4 different pyruvate dehydrogenase kinases (PDK1-4) and 2 different pyruvate dehydrogenase phosphatases (PDP 1-2), which are differentially expressed in mammalian tissues. Although PDHα has three phosphorylation sites, phosphorylation of site 1 (S293, in the immature rodent and human PDHα protein) reduces overall PDC activity by >97% (24). Since PDC regulation determines the rates of pyruvate oxidation, and therefore the relative ratio of glycolytic vs. oxidative glucose metabolism in cells, it is surprising that relatively little is known about the differential expression of PDC components and regulatory proteins in astrocytes vs. neurons. Moreover, the potential role of differential PDHα phosphorylation in supporting the astrocyte-neuron lactate shuttle remains unexplored. Here we provide evidence supporting a role for differential PDC activity in distinguishing glucose metabolism patterns between astrocytes and neurons and in coordinating the corporate metabolism of glucose among brain cells.

Results

Enriched Astrocyte and Neuronal Cultures Display Unique Metabolic Profiles

We utilized cerebrocortical cultures of rat astrocytes and neurons in this study. The purity of the cell cultures was assessed by the expression patterns of the astrocyte specific intermediate filament GFAP, and the neuronal specific microtubule stabilizing protein MAP2 in cell extracts (Fig. 1). Figure 1A and 1B show that the majority of cells in the respective cultures predominantly express characteristic neuronal or astrocytic

morphological features and markers. With prolonged exposure times, only a very weak GFAP signal could be detected in neuronal cultures. Based on microscopic counts of $GFAP^+$ or $MAP2^+$ cells, the respective cultures were both estimated to be >95% pure as shown previously (28, 29).

Both astrocytic and neuronal cultures expressed LDH as demonstrated by Western blotting using a polyclonal antibody to both isoforms (LDH-A and LDH-B, Fig. 1B). However, as shown by blot densitometry (Fig. 1C), LDH protein expression was significantly higher in astrocyte cultures than in neuronal cultures ($p < 0$ .0001). LDH enzyme activity, normalized to total protein, was also much higher in astrocytes (Fig. 1D; $p < 0$ .0001) and was consistent with a higher lactate production by astrocytes than by neurons (Fig. 1E). Using live cell NAD(P)H fluorescence imaging (Fig. 1F) we observed prominent fluorescence in both cell types. However, two different patterns could be readily discerned. A strong NAD(P)H fluorescence signal in both cell types was similar in distribution to the immunofluorescence staining pattern of mitochondrial PDHα (Fig. 1F). A diffuse fluorescent NAD(P)H signal was also observed in the cytosol and nucleus. Quantification of the of NAD(P)H fluorescence revealed a significantly higher ratio of nuclear to mitochondrial fluorescence in astrocytes compared to neurons (Fig. 1G; $p = 0$ .0002). Since the non-mitochondrial NAD(P)H fluorescence largely reflects glycolytic activity (9), these results are consistent with astrocytes metabolizing glucose to pyruvate at a much higher rate than neurons.

Astrocytes and Neurons Display Distinct Expression Profiles for the Pyruvate Dehydrogenase Complex, PDH Kinases and PDH Phosphatases

Having observed PDHα immunostaining in both astrocytes and neurons, we sought to determine if other PDC components were expressed in each cell type. All subunits of the PDC could be detected in both neuron and astrocyte cultures (Fig. 2A). We observed a slight shift in the mobility of PDHα during SDS-PAGE between astrocytes and neurons (Fig. 2A and 3D). This shift was observed using four different antibodies, and suggested the possibility of different PDHα isoforms occurring in the two cell types. However, when the PDC complex was immunoprecipitated from the two cell types using an antibody to the E2 subunit and was then probed by Western blot with the PDHα antibody, the difference in molecular weights disappeared (Supplementary Figure S1). These data suggest that other abundant proteins in the crude cell extracts affect the apparent mobility of PDHα in the gel, causing an apparent difference in molecular weight. Surprisingly, when normalized to the same level of total protein, astrocytes had significantly higher levels of immunoreactivity for all subunits of the PDC, but particularly for DLD (E3/E3bp) and PDHβ. Densitometric analysis of Western blots showed that astrocytes expressed significantly higher levels of PDHα ($p = 0.034$), PDHβ ($p = 0.02$), DLD ($p < 0.001$), and DLAT ($p < 0.001$). In contrast, immunoreactivity for subunit IV of the mitochondrial cytochrome oxidase enzyme complex (COX IV) was equal in both cultures ($p = 0.84$) (Fig. 2A).

We could detect immunoreactivity for all known mammalian PDKs (PDK1-4) and PDPs (PDP1, 2) in both astrocyte and neuron cultures. However, unique expression levels of these proteins were noted in the two cell types (Fig. 2B). Blot densitometry showed astrocytes to have significantly higher immunoreactivity for PDK2 ($p <0 .0001$) and PDK4 ($p <0 .0001$). Neuronal cultures expressed significantly higher levels of PDK1

than astrocytes ($p = 0.03$), although the differential expression of PDK1 between astrocytes and neurons was less than that for PDK2 and PDK4. No significant difference in immunoreactivity for PDK3 ($p = 0.93$) was observed between the two cell types. Cultures of astrocytes and neurons also expressed both PDP1 and PDP2 (Fig 2B). However, immunoreactivity of PDP1, the Ca^{2+} sensitive phosphatase, was significantly higher in astrocyte cultures ($p < 0.0001$), while the immunoreactivity of PDP2, the Ca^{2+} insensitive phosphatase, was similar in both cultures ($p = 0.23$).

Astrocytes Display Higher PDHα Phosphorylation and Lower PDC Activity than Neurons

Most studies examining PDHα phosphorylation have utilized ^{32}P incorporation approaches which are sensitive to many experimental variables and do not reflect the true endogenous phosphorylation status. In order to trap and directly assess the phosphorylation status of the PDHα protein, we generated a phospho-specific antibody that specifically recognizes the phosphorylation of site 1 (serine 293). Using recombinant PDH, the antibody was found to recognize a protein containing phospho-serine 293 but had little to no cross-reactivity with the recombinant unphosphorlyated PDHα (Fig. 3A). To demonstrate the specificity of this antibody in tissue extracts we performed an *in vitro* PDHα phosphorylation assay in isolated rat whole brain mitochondria using ^{32}P-γATP. Since the PDKs and PDPs are contained inside isolated intact mitochondria, the phosphorylation of PDHα can be easily manipulated in such preparations (30). Autoradiographic analysis of SDS-PAGE separated proteins transferred to nitrocellulose membranes revealed the most prominent ^{32}P-labeled band to

be at approximately 42 kDa. Treatment with Mg^{2+}, which is required for PDP activity, greatly reduced labeling of this band (Fig. 3B, top panel). Subsequent immunoblotting of the same membrane with anti-PDHα ^{pS293} or anti-PDHα demonstrated Mg^{2+}-induced dephosphorylation of PDHα when the PDHα ^{pS293} targeted antisera was employed. This reduction in immunoreactivity was not seen with PDHα targeted antisera (Fig. 3B). To further confirm the specificity of the PDHα ^{pS293} antisera we performed two-dimensional electrophoresis on rat brain mitochondrial protein extracts. Subsequent immunoblotting of separated proteins produced a train of spots, characteristic of phosphoproteins, at the molecular weight (42 kDa) and isoelectric point (pI) observed for PDHα (30) (Fig. 3C). To determine the spotting differential of the PDHα ^{pS293} antibody to the total PDHα antibody we stripped the membrane and then re-probed it using a mouse monoclonal anti-PDHα antibody. Immunoblotting with anti-PDHα produced a similar train of spots at a similar MW and pI as the PDHα ^{pS293} antibody, although one more spot was observed (Fig. 3C, see arrow).

Using the PDHα ^{pS293} antibody we next discovered that astrocyte cultures had significantly higher levels of PDHα ^{pS293} ($p < 0.001$) than neurons. Moreover, the ratio of PDHα ^{pS293} to PDH E1α ^{total} was much higher in astrocytes compared to neurons ($p = 0.02$) (Fig. 3D, E), suggesting that PDC might be less active in astrocytes than in neurons. In order to determine whether the differential PDC activity was linked to the differential phosphorylation status of PDHα in the two cell types, we utilized dichloroacetate (DCA), an established inhibitor of PDKs when used at mM concentrations. Using isolated whole brain mitochondria we were able to demonstrate a dose dependent inhibition of PDHα phosphorylation with DCA (Fig. 4A). When tested on live astrocytes and neurons

however, DCA showed dose-dependant toxicity to neurons in its effective dose range (Fig. 4B). Surprisingly, isolated astrocytes displayed no toxicity, even when cultured for 36h in 10mM DCA, despite the continuous inhibition of PDHα phosphorylation over this time (Figure 4B). Use of this pharmacological approach thus precluded a comparative analysis of reversible PDH phosphorylation between live neurons and astrocytes. However, DCA treatment was clearly able to lower lactate production by astrocytes dose-dependently, down to the level seen in neurons (Figure 4D).

In order to directly determine whether PDC activity was indeed higher in neurons despite higher overall expression of the complex proteins in astrocytes, we used a commercial assay that combines immunoprecipitation with quantification of PDC protein and enzyme activity. This assay revealed that neurons have more than 50% higher specific activity of the PDC than astrocytes (Fig. 5A). To directly relate PDHα phosphorylation status to the differential PDC activity observed between astrocyte and neuronal lysates, we then assayed PDC activity with and without addition of recombinant active PDP-1 to force complete dephosphorylation of PDH α (31). Neurons were again found to have high PDC activity that was stimulated ~25% after PDP-1 treatment. Astrocyte displayed much lower PDC activity compared to neurons but this activity was stimulated >300% after PDP-1 treatment (Fig. 5B & C). Thus, native PDC was found to be operating at close to its maximal activity in neurons but far from its maximal activity in astrocytes. Together these data support a role for PDHα phosphorylation in determining the differential metabolic phenotype of astrocytes and neurons.

Discussion

The aim of the present study was to elucidate the role of the PDC in the differential metabolism of pyruvate/lactate in primary cultures of rat cerebral cortical astrocytes and neurons. We hypothesized that regulation of the PDC at either the protein expression, protein phosphorylation, or enzyme activity level contributed to the differential metabolic phenotype of neurons and astrocytes and to the directional shuttling of monocarboxylates between these cell types. We demonstrated for the first time that all of the subunits of the PDC are expressed in cultured astrocytes and neurons but that astrocytes surprisingly express significantly higher immunoreactivity for all subunits compared to neurons (Fig. 2A). By contrast, immunoreactivity for COX IV, a component of the oxidative phosphorylation complex is similar in both cell types. PDC activity is largely regulated by reversible phosphorylation of PDHα (24, 32) and using isozyme specific antibodies we show for the first time that the PDH kinases and phosphatases are differentially expressed between astrocytes and neurons. Control of PDK expression levels is an established means for cells and tissues to regulate PDC activity and thus glucose oxidation rates (33). The higher expression of PDK2 and PDK4 in astrocytes vs. neurons is thus consistent with the higher PDHα phosphorylation status, lower PDC activity, and higher lactate production displayed by astrocytes. Higher LDH immunoreactivity was also observed in astrocytes using a polyclonal antibody to both LDH isozymes (LDH-A and LDH-B) (Fig. 1B) as well as higher LDH activity. This observation, as well as the higher non-mitochondrial to mitochondrial ratio of NAD(P)H in astrocytes strongly supports the pervading notion of astrocytes being highly glycolytic cells.

The predominant expression of PDP-1 in astrocytes is surprising. PDP-1 activity strongly depends on the presence of calcium while PDP-2 is believed to be calcium

independent (34). Regulation of PDH activity in brain slices has previously been attributed to changes in calcium accumulation in neurons (35). Our results suggest that astrocytes may have contributed significantly to these prior observations. Since many neurotransmitters increase astrocytic calcium levels (36), it is possible that astrocytic PDC activity may be regulated by intra- and intercellular signals via changes in PDHα phosphorylation. The overall increased expression levels of PDC regulatory enzymes in astrocytes are consistent with a tighter regulation of pyruvate (and lactate) metabolism in this cell type. Tight control of PDHα phosphorylation status in cultured astrocytes may ensure pyruvate being blocked from entry into the Krebs cycle and being converted instead to lactate via LDH activity. Since transport of lactate (and pyruvate) through MCTs is bidirectional and dependant on concentration gradients of the monocarboxylates and H^+ (37-39), astrocytic PDC activity status may contribute significantly to the directional shuttling of monocarboxylates from astrocytes to neurons. The dephosphorylation of PDHα and lowering of astrocytic lactate production by DCA is consistent with this notion. Moreover, the strong activation of PDC in astrocytic extracts through forced dephosphorylation of PDHα suggests a novel potential mechanism for varying the efficiency of astrocytic-neuronal metabolic coupling. Thus under the appropriate conditions, astrocytes may be able to switch from being glycolytic to glucose oxidizing cells (18). Moreover, as in other cell types, changes in PDH phosphorylation may allow astrocytes to switch between oxidation of glucose to other fuels that bypass PDH (21). Neurons, on the other hand appear to maintain high PDC activity due to low PDHα phosphorylation levels, thus remaining poised for and committed to pyruvate oxidation. Overall our findings support a prominent role for astrocytes in controlling

neuronal metabolism. Astrocytes may also be far more flexible with respect to metabolic routing of fuel substrates than neurons.

Material and Methods

Materials

Chemicals, antibodies and other reagents were purchased from the following sources: fetal bovine serum, DMEM, B-27 supplement, neurobasal media, penicillin/streptomycin, mouse anti-PDHα (1:1000), Zoom® 2D Protein Solubilizer #1, NuPAGE® LDS Sample Buffer, NuPAGE® Sample Reducing Agent, and mouse anti-COX IV, prolong antifade with DAPI, and fluorescently labeled secondary antibodies were purchased from Invitrogen (Carlsbad, CA). BCA™ Protein Assay Kit – Reducing Agent Compatible was purchased from Pierce (Rockford, IL). Mouse anti-PDHβ (1:1000) and mouse anti-DLAT/DLD (E2/E3 subunits, 1:1000) antibodies and MitoProfile Assay kit for PDC activity were purchased from Mitoscience (Eugene. OR). Rabbit anti-PDK1 (1:1000) and PDK3 (1:3000) were obtained from Stressgen (Ann Arbor, MI) and Abgent (San Diego, CA), respectively. Rabbit anti-PDP1, PDP2 and mouse anti-PDK2 and PDK4 were generated in laboratory of Dr. R.A. Harris (40, 41). Rabbit anti-glial fibrillary acid protein (GFAP) and mouse anti-microtubule associated protein-2 (MAP2) were purchased from Chemicon (Temecula, CA) and Sigma-Aldrich (St. Louis, MO) respectively.

Generation of antibody specific to phosphorylated site 1 of PDHα

A pS293 phospho-specific antibody was generated in New Zealand white rabbits by injecting a peptide (YRYHGH(pS)MSDPG) (sequence of the precursor human and rodent protein) conjugated to the adjuvant KHL (Novus Biologicals). The resulting serum was then negatively purified by preabsorption against unphosphorylated peptide. To determine the phospho-serine293 specificity of the antisera, human recombinant PDHα, either phosphorylated or unphosphorylated at serine 293, was separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted (as described below) using the antisera described above.

Brain Mitochondria Isolation

Brain mitochondria were isolated from adult rats by sucrose centrifugation as described previously (42). Mitochondria were assayed for total protein content by the Bradford method and aliquots were stored at -20°C.

^{32}P-γATP Labeling

To evaluate the specificity of the rabbit anti- PDHα ^{pS293} antibody, *in vitro* phosphorylation of brain mitochondria was accomplished by incubation with 6 μCi/mmol ^{32}P-γATP alone, or with the addition of 10 mM $MgSO_4$ for 30 min at 37°C. The reaction was terminated by the addition of sample buffer. The proteins were resolved by SDS-PAGE, transferred to nitrocellulose and subsequently evaluated for *in vitro* phosphorylation by autoradiography, or immunoblotting for PDHα ^{pS293} and PDH.

Cell Cultures

Neuron primary cultures were obtained from rat fetuses extracted by caesarian section from timed 17-day pregnant Sprague-Dawley female rats (Taconic; Albany, NY). Fetal brains were removed and placed in Hanks balanced salt solution supplemented with Penicillin/Streptomycin. Cortices were dissected and meninges removed, and then tissue was minced and enzymatically digested with trypsin and DNase. Following digestion cells were further triturated then passed through a cell strainer. Cells were plated onto poly-D-lysine-treated plates in DMEM containing 9% FBS and 1% Penicillin-streptomycin at a density of 234,000 cells/cm^2. After 24 hours the media was replaced with Neurobasal A media containing 2% 50X B27 supplement, 1% Penicillin-streptomycin, and 0.5mM glutamine. Half of the media was replaced every third day for the duration of the experiments. Primary glia cultures from the cerebral cortex highly enriched in astrocytes were isolated as described previously (43).

Protein Isolation and Immunoblotting

After 7 or 10 days, for neurons or astrocytes respectively, the media was removed and the cells were rinsed with PBS. Proteins were extracted using Zoom 2D Protein Solubilizer #1 as described by the manufacturer. Protein concentrations were determined using the BCA protein assay kit. Homogenates containing 80 μg of total protein were prepared for immunoblotting by diluting samples with water, NuPAGE® LDS Sample Buffer, and NuPAGE® Sample Reducing Agent to final volume of 20 μl. Samples were then heated to 70°C for 10 minutes to denature proteins. Neuron and astrocyte samples were loaded onto precast 10% Bis-tris polyacrylamide gels and separated by electrophresis at 190 V. Following electrophoresis proteins were transferred onto

nitrocellulose membranes (35 V for 4h) prior to immunoblotting. Membranes were blocked for 2h in 10% normal serum (or 5% non-fat milk for LDH) in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBS-T), then incubated with primary antibodies overnight at 4°C. Blots were rinsed in TBS-T, incubated with the appropriate peroxidase-conjugated secondary antibodies for 2h in 5% normal serum in TBS-T. Blots were developed in ECL-plus and exposed to film. Films were digitized and the resulting images were analyzed using ImageJ software.

Two Dimensional Gel Electrophoresis

Rat brain mitochondria were isolated and the protein was extracted as described above. 2D-electrophoresis was performed on rat brain mitochondria using the Zoom® bench top proteomics system according to the manufacturer's protocol. Briefly, homogenates were loaded onto pH 3-10 IPG strips according to the manufacturer's instructions. IPG strips were then separated using a step-voltage protocol (175 V, 15 min; 175-2000 V, 45 min; 2000 V, 45 min). IPG strips were then removed treated with NuPAGE® LDS Sample Buffer with NuPAGE® Sample Reducing Agent, then NuPAGE® LDS Sample Buffer with 125 mM iodoacetamide prior to separation in the second dimension. SDS-PAGE, transfer of proteins, and immunoblotting were preformed as described below.

Immunocytochemistry

Cells grown on glass coverslips were fixed on ice in cold (-20°C) 70% acetone/30% methanol for 8 minutes then rinsed thoroughly in PBS. Coverslips were

incubated in 2% fish skin gelatin in PBS at room temperature for 1 hour, then incubated in 1% fish skin gelatin in PBS with primary antibodies at 4°C overnight. After through rinsing in PBS sections were incubated with appropriate fluorescent-conjugated secondary antibodies and mounted using Prolong anti-fade with DAPI.

Biochemical Assays

Neuronal and astrocyte cultures were assayed for specific activity of the PDC using a commercially available MitoProfile® microplate assay kit. This kit consists of an assay for PDC quantity (a PDC ELISA) and activity (spectrophotometric measurements of NADH production). The assays were performed according to the manufactures guidelines; the results of the both assays were then used to determine specific activity of the PDC (PDC activity/PDC quantity) and expressed as a mean of astrocyte specific activity. PDC activity was determined as described previously (31). LDH activity was determined using a Hitachi-747 clinical analyzer and expressed as activity (units/liter) normalized to total protein per sample (units/liter/mg total protein). Media lactate levels were assessed using a commercial CMA 600 microdialysis system. Lactate levels were expressed as mM lactate per 10^6 cells (mM/10^6 cells)

NAD(P)H Imaging

Cells grown on coverslips were imaged with a Zeiss Axiovert 200M microscope equipped with an Exfo X-cite light source and a 40 X oil objective (N.A.=1.3). Excitation light intensity was reduced 90% with a neutral density filter to reduce photobleaching. Excitation light passed through an 11nm bandpass filter centered at 387

nm (Semrock, Rochester, NY). The dichroic cutoff was 409 nm, and the emission window was 80 nm, centered at 460 nm. Cells were imaged in Hepes-buffered Krebs' buffer (with 5 mM glucose) inside a chamber kept at 37°C. Images were collected with a Hammamatsu Orca ER CCD camera with the binning set to 4, with acquisition controlled by Volocity. ImageJ was used to demark regions of interest and the average pixel intensity of mitochondrial and nuclear fluorescence was expressed as a ratio. Mitochondrial fluorescence was attributed to PDC activity, while nuclear fluorescence was attributed to NADH derived from glycolysis or LDH (with the assumption that nuclear and cytosolic NADH are in rapid equilibrium through the nuclear pore complex).

Statistical Analysis. Two-tailed Student's *t*-tests were used to determine pair-wise differences between neuron and astrocyte cultures.

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Figure Legends

Fig. 1. Astrocyte and neuronal cultures were characterized by (*A*) phase contrast imaging, (*B*) astrocyte-specific (GFAP) or neuron specific (MAP-2) markers or LDH immunoreactivity, (*C*) densitometry of LDH blots, (*D*) LDH activity and (*E*) lactate release by astrocytes (dark bars) and neurons (light bars). (*F*) Fluorescence imaging of cell-specific immunochemical markers (MAP-2, green; GFAP, red; DAPI, blue), NAD(P)H, and PDH alpha immunocytochemistry (red; DAPI, blue). (*G*) Nuclear to mitochondrial imaging of NAD(P)H fluorescence in astrocytes (dark bars) or neurons (light bars). Astrocytes and neurons are labeled as A or N respectively. Data presented here are the result of quadruplicate Western blot measurements from three independent cultures.

Fig. 2. Pyruvate dehydrogenase complex (PDC) subunit expression in astrocytes and neurons. Astrocyte and neuronal cell extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes and immunostained for (*A*) PDHα, PDHβ, DLAT (E2), DLD (3/E3bp), COX IV and (*B*) PDK1-4 and PDP1-2. Data presented here are representative of quadruplicate Western blot measurements from three independent cultures.

Fig. 3. Characterization of phopshospecific antibody to serine 293 of PDHα. (*A*) Following gel separation and transfer to membrane, recombinant human PDHα and

PDHα containing phosphorylated serine 293 were immunostained with anti-phosphoserine 293 antiserum (*B*) Rat brain mitochondria were incubated with ^{32}P ATP in the absence or presence of 10mM Mg^{2+} or 1mM EDTA for 30 min, solubilized in sample buffer, subjected to SDS-PAGE and transferred to membrane. The blot was exposed to film to generate an autoradiogram followed by immunostaining using anti-phosphoserine 293 antiserum. The same blot was stripped and probed again using a monoclonal antibody to PDH alpha. (*C*) Rat brain mitochondria were subjected to 2D-gel electrophoresis, membrane transfer and immunoblotting with anti-phosphoserine 293 antiserum. The same blot was stripped and probed again using a monoclonal antibody to PDHα. (*D*) Astrocyte and neuronal cell extracts were subjected to gel electrophoresis, membrane transfer and immunoblotting with anti-phosphoserine 293 antiserum. The same blot was stripped and probed again using a monoclonal antibody to PDHα. (*E*) Immunoblots of astrocyte and neuronal cell extracts were analyzed by densitometry and expressed as the ratio of phospho-PDH alpa to PDH alpha. Data presented here are representative of quadruplicate Western blot measurements from three independent cultures.

Fig. 4. Effect of dichloroacetate on PDH phosphorylation, cell viability, and lactate production. *(A)* Brain mitochondria incubated with indicated DCA concentrations were processed for Western blotting using anti-phospho PDHα and anti-PDHα antisera. *(B)* Neuronal and astrocytic cultures were incubated with indicated DCA concentrations for 1 hour and assessed for cell viability by cell counting. *(C)* Astrocytes incubated with 10 mM DCA for 1 hour were analyzed for PDHα phosphorylation via Western blotting

using anti-phospho PDHα and anti-PDHα antisera. *(D)* Lactate release into the extracellular media over 1h was measured in astrocytes treated with the indicated concentrations of DCA and compared to untreated neurons. Bar heights and error bars represent mean concentrations +/- SEM of media lactate determined in three experiments from four independent cultures. (* $p < 0.05$)

Fig. 5. Effect of PDC dephosphorylation on PDC activity in astrocyte and neuronal extracts. *(A)* Specific activity of the PDC determined in astrocyte and neuronal lysates following PDC immunocapture. *(B, C)* PDC activity determined in astrocyte and neuronal extracts before and after addition of recombinant PDC phosphatase (PDP). Absolute activity shown in (B) is expressed as percent maximal activity for each cell type in (C). Bar heights and error bars represent mean enzyme activity rates +/- SEM determined in experiments from four independent cultures of astrocytes or neurons. (*$p < 0.001$)

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Figure 1

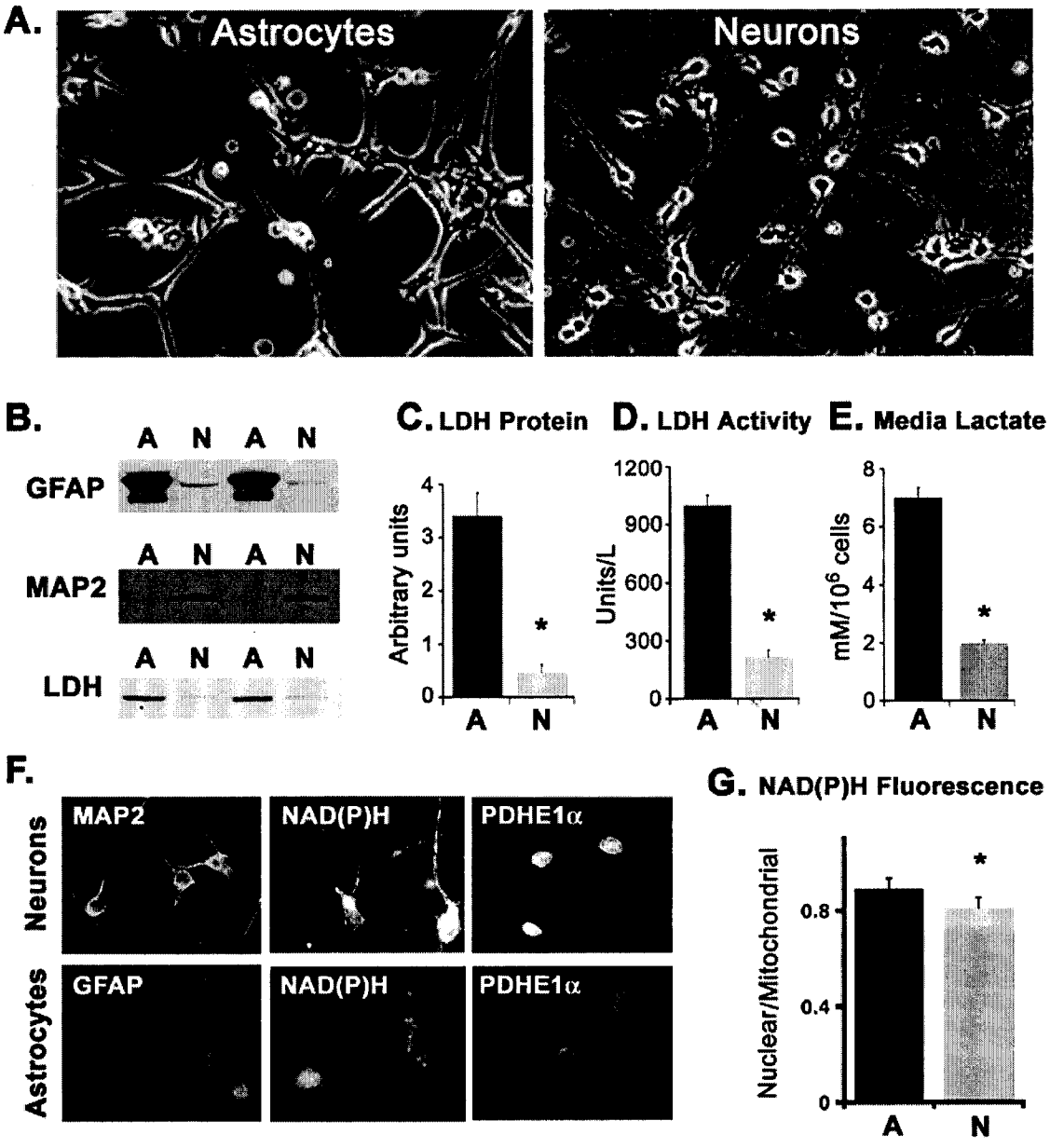

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Figure 2

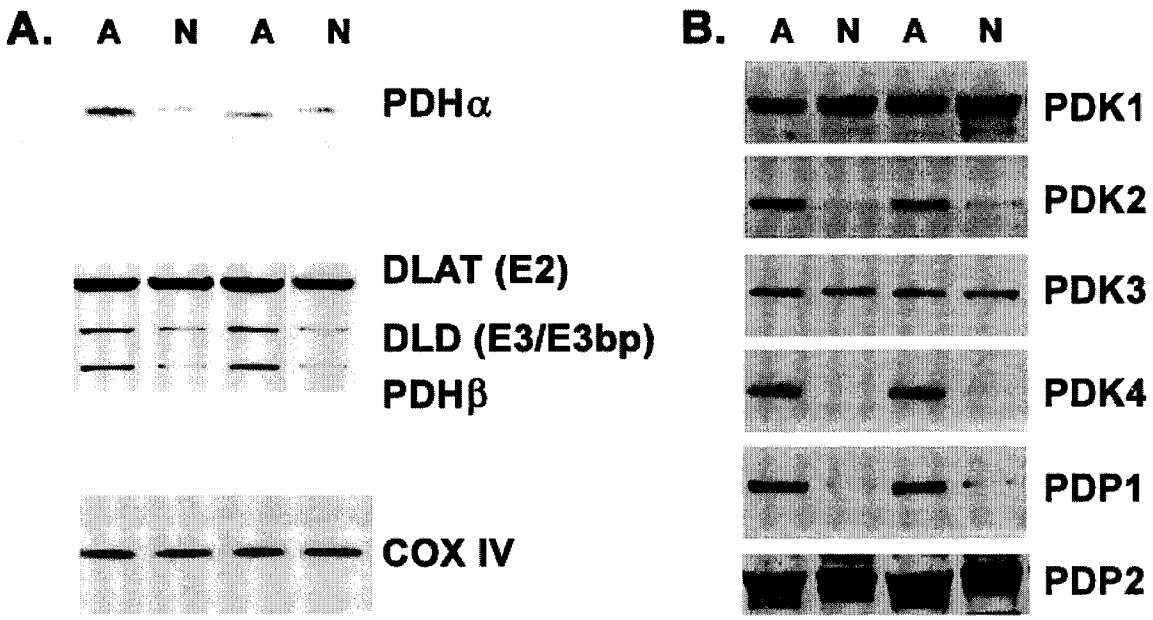

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Figure 3

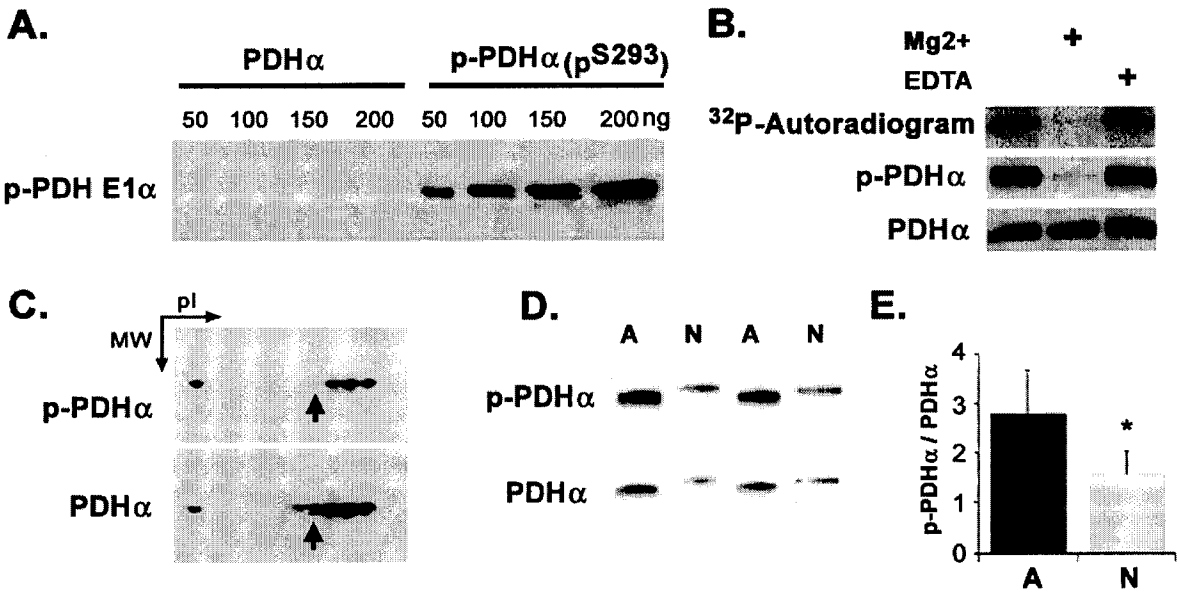

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Figure 4

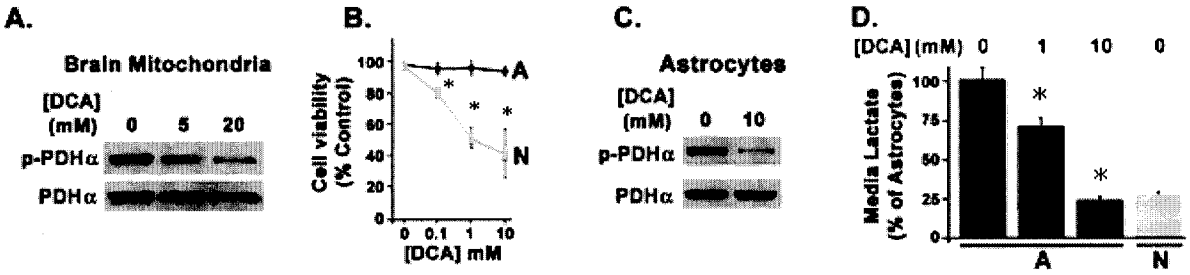

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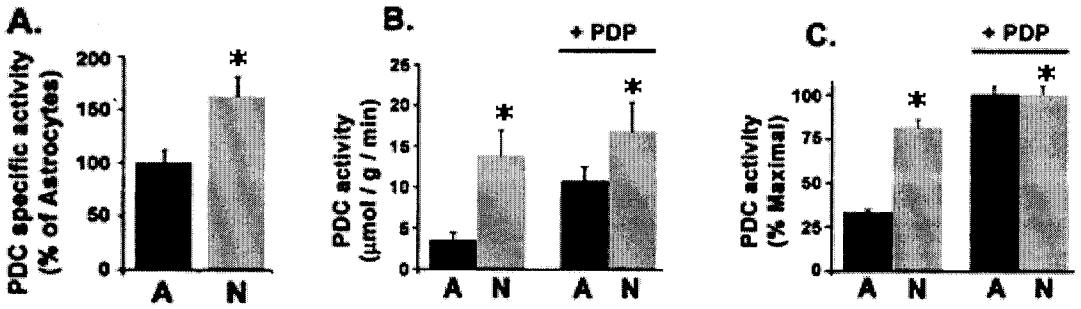
Figure 5



Supplementary Figure 1

Chapter 3 Significance and Summary

The proceeding chapter proposes that antipsychotic drug administration results in an increase in postmortem brain lactate levels. This is demonstrated in rodents chronically treated with haloperidol and clozapine. We also demonstrate significantly higher levels of lactate in the postmortem cerebellum of patients with schizophrenia relative to normal controls. This observation is in line with a previous finding of an increase in postmortem lactate levels in the prefrontal cortex of patients with schizophrenia. In light of our rodent data we propose that increases in postmortem brain lactate levels are the result of antipsychotic administration and a secondary effect of the disease not a primary feature of schizophrenia.

These data highlight a key issue in postmortem brain research, that is, what effects are primary to the disease and what are secondary confounding effects. As a population patients with schizophrenia have higher rates of smoking, substance misuse, and social isolation all of which can alter the biology of the brain. Furthermore these patients are normally treated with several different classes of neuropsychiatric medications. These effects must be accounted for in postmortem brain research as they are likely to induce numerous alterations which may be falsely attributed as a primary feature of the disease.

Chapter 3: Postmortem Brain Lactate Levels in Patients with Schizophrenia and Rats Chronically Treated with Antipsychotics

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Abstract

In neuropsychiatric brain collections, a lower brain pH is often observed in tissues from patients with schizophrenia. Recently, studies have identified metabolic abnormalities (i.e. increased lactate concentrations and altered transcription of metabolic genes) in patients with schizophrenia, and proposed that these changes may underlie or contribute to the pathophysiology of the disease. As antipsychotic drugs produce a wide variety of metabolic and molecular changes, we hypothesized that increased lactate levels and decreased postmortem pH in patients with schizophrenia are the result of drug-induced alterations and not primary features of the disease. We measured lactate levels in the cerebellum of patients with schizophrenia (n = 35) and control subjects (n = 42) and in rats chronically treated with haloperidol (0.8 mg/kg; n = 5), clozapine (5 mg/kg; n = 5), or vehicle (0.1% lactic acid; n = 5). We found a significant increase in lactate levels in samplsfrom patients with schizophrenia as well as in samples from rats treated with clozapine and haloperidol. These data suggest that alterations in lactate levels may be a generalized phenomenon in the brains of patients with schizophrenia and that this effect could be the result of antipsychotic treatment.

Introduction

Postmortem brain research is an important tool in elucidating the pathophysiology of neurological and neuropsychiatric conditions. Postmortem tissue can be used in a wide variety of molecular, biochemical, and cellular research; thus, it represents a unique resource to investigate disease-induced alterations. The interpretation of the results of these studies must be evaluated carefully as the observed changes may be primary to the disease or may be the secondary effects of disease or medication.

A common feature of brain tissue from multiple cohorts of schizophrenic patients is a decreased brain pH relative to controls [11, 24, 30, 38]. The significance of this phenomenon is unclear. It may arise from differences in the manner of death between patients with schizophrenia and controls, premortem acidosis, medication-induced alterations, or it may reflect a primary feature of schizophrenia.

Recent studies have identified alterations in metabolic genes or a variety of metabolites in the brain as well as in other tissues in patients with schizophrenia [30]. The results of one of these studies found altered transcription of metabolic genes and increased lactate levels in the prefrontal cortex (PFC) of patients with schizophrenia. The authors hypothesized that these abnormalities constitute the "vulnerability" of the PFC and when combined with a variety of genetic and/or epigenetic factors result in the deficits that characterize schizophrenia. The authors argued that decreased pH and increased lactate levels are not postmortem artifacts but may underlie the pathophysiology of schizophrenia.

As antipsychotic treatments induce numerous changes in metabolism both *in vitro* [12, 27, 39] and *in vivo* [7, 10, 28], we hypothesized that increased lactate concentrations

observed in postmortem brains of patients with schizophrenia are the result of antipsychotic administration and not a primary feature of the disease. To elucidate the significance of increased lactate concentrations in the postmortem brains of schizophrenic patients, we performed two studies: we investigated whether increased lactate concentrations were found in the cerebellum, a region of the brain not commonly associated with schizophrenia, and whether postmortem brain lactate levels were increased in rats chronically treated with haloperidol or clozapine. In this study, we found that lactate levels were increased in the cerebellum of patients with schizophrenia and that chronic administration of clozapine and haloperidol increased lactate levels in the frontal cortex of rats.

Materials and Methods

Human Subjects

Human brain specimens were collected in the Section on Neuropathology of the Clinical Brain Disorders Branch at the National Institute of Mental Health (NIMH) through the Offices of the Chief Medical Examiner of the District of Columbia and of Northern Virginia, after autopsy, and through tissue donations via funeral homes. Informed consent to study brain tissue was obtained from the surviving next-of-kin for all cases, according to Protocol #90-M-0142 approved by the NIMH/National Institutes of Health Institutional Review Board. A telephone interview with the next-of-kin to gather basic demographic information and medical, substance use, and psychiatric history was conducted within 1 week of donation. Detailed information regarding diagnosis,

antipsychotic medication history, neuropathology, and other information is described elsewhere [24] and summarized in Table 1.

Drug Preparation

A stock solution of haloperidol (Sigma Chemicals, St Louis, MO) (20 mg/ml) was prepared by heating 200 mg of haloperidol in 10 ml 1% lactic acid until dissolved. To obtain a solution of 0.8 mg/ml haloperidol, the stock solution was diluted with distilled water and NaOH (1 N) was added to adjust the final solutions to a pH of 5.1. Clozapine (Sigma Chemicals) was prepared daily by dissolving 140 mg of clozapine in 0.6 ml of 1 N HCl with gentle heating, then diluting the solution with distilled water to 5 mg/ml and neutralized with 1N NaOH to a pH of 5.1. Vehicle consisted of 0.1% lactic acid.

Animals and Drug Administration

Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) (n=15, weight 225-250 g) were housed two per cage with *ad libitum* access to food and water. All procedures were performed in accordance with the National Institutes of Health Guide for Use and Care of Laboratory Animals. After a 1-week habituation period, animals were administered haloperidol 0.8 mg/kg, n=5), clozapine (5 mg/kg, n=5) or vehicle (n=5) daily via intraperitoneal (i.p.) injections. This dose regimen was chosen to emulate the therapeutic range of doses given to patients [19], and was showed to be effective in our previous behavioral and biochemical studies [1, 25, 26]. All animals were administered daily injections of drug or vehicle for 4 weeks. Rats were killed by decapitation, the brains were quickly removed, frontal cortex dissected and quickly frozen on dry ice.

Tissue Preparation and Lactate Measurements

Rat frontal cortex (1 g tissue : 10 ml protease inhibitors-Tris-glycerol) and human cerebellum tissue samples (1 g tissue : 10 ml PBS-protease inhibitors) were thoroughly homogenized then centrifuged at 16,000 *g* for 20 minutes. Supernatant lactate measurements were obtained by a standard colorimetric method on a CMA 600 microdialysis analyser using a lactate reagent kit (CMA Microdialysis).

Statistical Analysis

Two-tailed Student's t-tests were used to examine if diagnostic groups (normal controls and schizophrenics) differed in variables such as brain pH, PMI, and age. Spearman's coefficients of correlation were calculated to examine if lactate levels were associated with age, pH, PMI, or any measure of antipsychotic treatment (i.e. daily, lifetime, or last chlorpromazine equivalents). A one-way ANOVA, followed by Fisher PLSD *post hoc* tests were used to test the effects of haloperidol and clozapine administration on lactate levels in the rat frontal cortex.

Results

Correlations with pH, PMI, age, and antipsychotic treatment

Cerebellar lactate levels correlated inversely with pH ($r = -0.53$, $p = 0.00001$; Fig. 2A) and weakly correlated with age at death ($r = 0.34$, $p = 0.06$; Fig. 2B). Lactate levels did not correlate with PMI ($r = 0.044$, $p = 0.44$; Fig. 2C) or any measure of antipsychotic

treatment in patients: last (r = 0.03; Fig. 3A), daily (r = 0.03; Fig. 3B), or lifetime doses (r = 0.14; Fig. 3C) expresses in CPZ equivalents.

Postmortem lactate levels in schizophrenia

Patients with schizophrenia had significantly higher levels of lactate in the cerebellum compared to controls (p = 0.00001; Fig. 1), and the pH of the cerebellar tissue was significantly decreased in patients (p = 0.02; Fig. 2A).

Lactate levels in rats treated with antipsychotics

ANOVA revealed a significant effect of antipsychotic treatment on lactate concentrations ($F_{(2,12)} = 11.1$, $p = 0.002$). *Post hoc* analysis revealed that both drugs, haloperidol at 0.08 mg/kg (p = 0.0005) and clozapine at 5mg/kg (p = 0.01), significantly increased lactate concentrations in the frontal cortex.

Discussion

The main finding of this study is increased lactate levels in the cerebellum, a brain region not commonly associated with schizophrenia, and increased lactate levels in the frontal cortex of antipsychotic-treated rats. Here we expanded a previous finding of increased lactate levels in patients with schizophrenia to the cerebellum [30]. In light of our animal studies we propose that this alteration is induced by antipsychotics and not a primary feature of a dysfunctional prefrontal cortex or schizophrenia.

Recent studies have identified transcriptional alterations in metabolic pathways in the hippocampus and in peripheral tissues (liver and blood) of patients with

schizophrenia [3, 31]. These postmortem studies (in the dorsolateral prefrontal cortex, hippocampus and liver) were conducted in a single cohort of patients, raising the possibility that these findings could be cohort-specific and not necessarily common to schizophrenia. Furthermore, in studies of red blood cells, the majority of schizophrenic patients were not drug naïve (n = 20 schizophrenic patients; 13 were drug-treated, 7 - drug naïve) [31]. These findings have lead to the emergence of a "metabolic" hypothesis of schizophrenia, yet it remains unclear whether the reported changes are primary to the disease or the result of epigenetic or medication-induced effects. Additionally, the results of these studies failed to identify an alteration common to these regions and/or tissues. A common feature found in multiple cohorts of schizophrenic subjects appears to be a decreased brain pH [11, 24, 30, 38].

Brain pH, along with other factors including agonal state and RNA integrity measures, is used as an indicator of tissue quality. [4, 13, 18, 24]. However, the cause and significance of decreased brain pH remains unclear as there is no perfect correlation between pH and RNA integrity and in many cases low pH does not necessarily predict poor RNA quality [24]. Some have speculated that this decrease is not a pre- or postmortem artifact but is reflective of the disease process, an underlying metabolic abnormality, oxidative stress [30], or even perhaps the higher rate of tobacco smoking in patients with schizophrenia [34]. In this study we demonstrated that pH is highly correlated with lactate levels and that increased lactate levels in patients with schizophrenia may account, at least in part, for the decrease in pH. We also demonstrated that chronic administration of haloperidol or clozapine increased lactate levels in the frontal cortex. This suggests that increased lactate levels, and to some extent, decreased

pH in patients with schizophrenia are medication-induced alterations and not primary to the disease. Indeed, antipsychotic administration can result in lactic acidosis [20] as well as ketoacidosis [9, 21-23, 35] which explain the decrease in brain pH of schizophrenics. Postmortem brain pH both in controls and schizophrenic patients is significantly lower than physiological pH. Medication induced lactic- or ketoacidosis might explain the differential decrease between schizophrenic and control subjects, but it does not explain the lower pH observed in control subjects. This is most likely the result of decreased oxygen intake and resulting hypoxia in the agonal state [37] resulting in acidosis and/or postmortem proteolysis.

Treatment with antipsychotics produces numerous metabolic alterations, including altered glucose metabolism, diabetes, and dyslipidaemia [2, 8, 14, 29]. Also, administration of a variety of psychotropic medication including lithium, antipsychotics, and tricyclic antidepressants can induce weight gain and obesity [33]. Aside from the increase in morbidity and mortality associated with obesity-related conditions, weight gain may decrease medication compliance making treatment less effective and promoting relapse [6]. Antipsychotic-induced weight gain is associated with both acute and chronic typical and atypical antipsychotic administration, though the effects are variable depending on the individual and the specific drug being administered [2].

Typical and atypical antipsychotics modulate numerous neurotransmitter systems and the exact therapeutic mechanism is unclear. The common therapeutic benefit of both classes of drug is thought to occur, at least in part, from their ability to antagonize dopamine signaling. Catecholamines regulate numerous aspects of metabolism including glucose utilization [5] and glycogen storage [36]. Lesions [15] and

drug [16] studies demonstrate that depletion of catecholamines results in increased glycogen levels in the brain. On the other hand, administration of amphetamine, a dopamine agonist, induces depletion of brain glycogen and this effect can be inhibited by administration of chlorpromazine [17]. Depletion of cerebral 5-hydroxytryptamine does not result in antagonism of amphetamine-induced glycogen depletion [16], suggesting that psychotropic mediated induction of weight gain may converge on catecholamine signaling. The increase in lactate levels in the cerebellum of patients with schizophrenia and in rats treated with antipsychotics may be the result of catecholamine antagonism and a resulting increase in glycogen stores. Aside from catecholamergic receptor modulation a recent report has suggested that atypical antipsychotics may increase food intake via H1 receptor mediated increase in AMPK signaling in the hypothalamus (CITE).

After death, glycogen stores are rapidly depleted and converted mainly to glucose and glucose 1-phosphate, and then to glucose 6-phosphate through the glycolytic pathway. Upon death, oxygen and NADH become depleted and oxidative phosphorylation ceases while the activity of glycolytic enzymes continues, until the levels of NAD^+ become limiting and glyceraldehydes 3-phosphate activity ceases. Lactate is a metabolic "dead end", that is, it cannot be further metabolized unless converted to pyruvate, but the reversible conversion of lactate to pyruvate is catalyzed by lactate dehydrogenase and requires reducing equivalents (NAD^+). Thus, at death, glucose as well as other glycolytic metabolites, are predominantly and rapidly converted to lactate.

We observed no correlation between any measure of antipsychotic administration (average lifetime, daily, or last dose exposed in chlorpromazine

equivalents) and lactate levels in schizophrenic patients (Fig. 3). These findings could be the results of several factors. Medical records may not be current and not reflective of specific antipsychotic or dose being administered. Chlorpromazine equivalents are extrapolated from patient medical records and thus may not reflect compliance at the time of death. Different classes of antipsychotics (i.e. typical or atypical) and other psychotropic medications may have varying biological effects [2]. Furthermore, chlorpromazine equivalents are based on antidopaminergic action and do not account for modulation of other neurotransmitter systems resulting in a incomplete picture of each specific drug's pharmacological profile [32].

The finding of elevated postmortem lactate levels in patients with schizophrenia and rats treated with antipsychotics could represent a drug-induced acidosis or more likely an alteration in glucose metabolism, such as increased glycogen levels, that result in increased postmortem lactate levels. Indeed, neither acidosis nor encephalopathy, the most commonly observed symptoms in patients with primary metabolic deficits are observed in patients with schizophrenia. Thus the findings of increased lactate levels in the brains of patients with schizophrenia may be due, at least in part, to antipsychotic treatment. Drug induced acidosis may account for lower postmortem pH observed in patients with schizophrenia. Further studies are required to elucidate the mechanism of antipsychotic induced glucose alterations.

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Table 1. Summary of Cohort Demographics

Patient (*n*)	Schizophrenia (35)	Control (42)	*t* test *p*-valve
Age (years)	52.5 (18.3)	45.8 (12.5)	0.06
Gender (M/F)	21/14	28/14	-
pH	6.38 (0.35)	6.58 (0.33)	0.02
PMI (hours)	35.6 (16.2)	32.9 (16.2)	0.44
Daily CPZ Equivalents	448	-	-
Last CPZ Equivalents	524	-	-
Lifetime CPZ Equivalents	3938291	-	-

Figure Legends

Figure 1. Scatter plot data of lactate levels (mmol) from the cerebellum of control ($n = 42$) and schizophrenic patients ($n = 35$). Lactate levels were significantly higher in patients with schizophrenia than control subjects ($p = 0.00001$)

Figure 2. Lactate levels are highly correlated to pH ($r = -0.53$), weakly correlated to age ($r = 0.34$), and not correlated to postmortem interval (PMI; $r = 0.04$).

Figure 3. Correlations of lactate to multiple measures of antipsychotic treatment. Lactate levels did not correlate to last (A), daily (B), or lifetime (C) chlorpromazine (CPZ) equivalents.

Figure 4. Lactate concentrations (mean SD) in the frontal cortex of rats treated with clozapine (5 mg/kg), haloperidol (0.8 mg/kg), or vehicle (0.1% lactic acid) for 28 days. Chronic intraperitoneal injections of haloperidol or clozapine significantly increased post mortem lactate levels relative to vehicle-treated animals ($*p < 0.01$ significantly different from vehicle control).

Figure 1

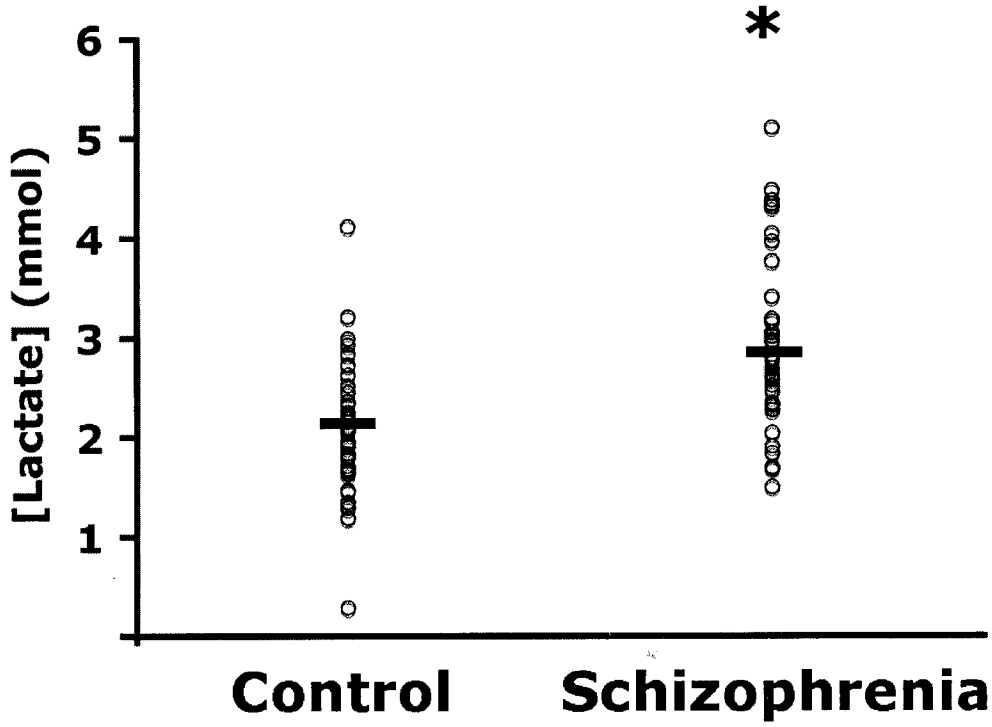

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Figure 2

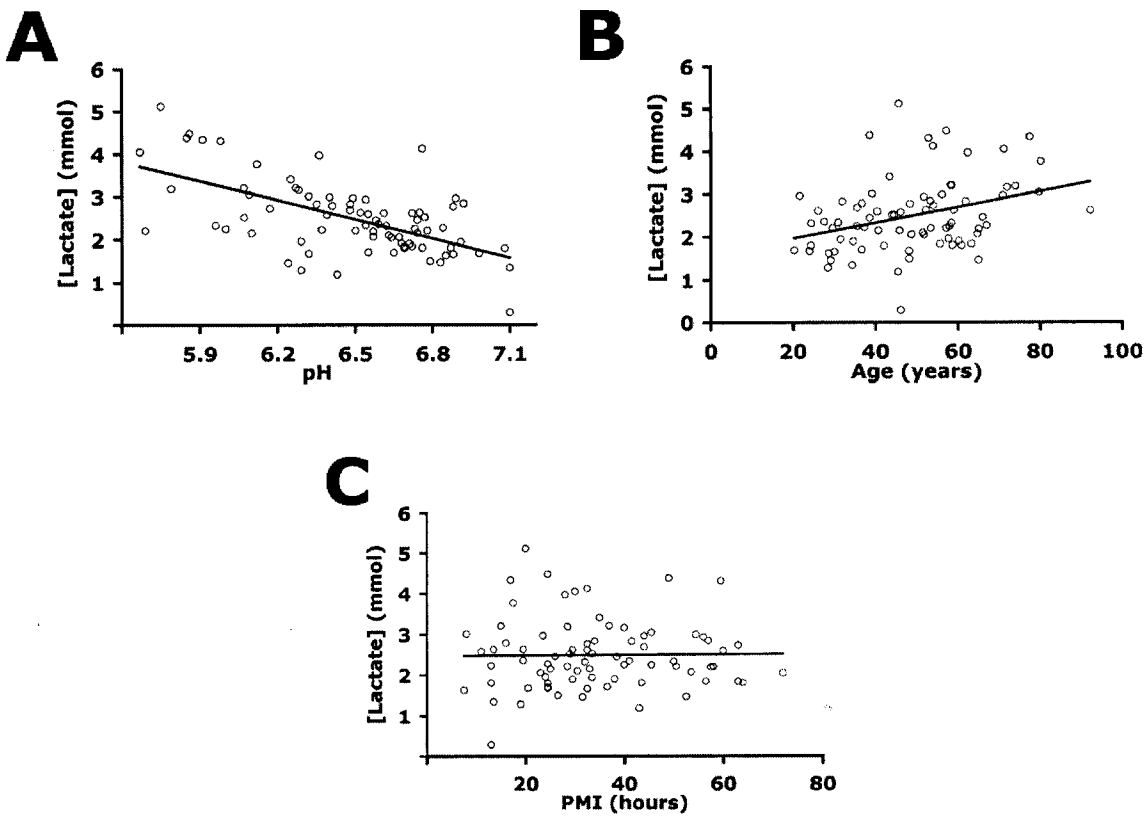

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Figure 3

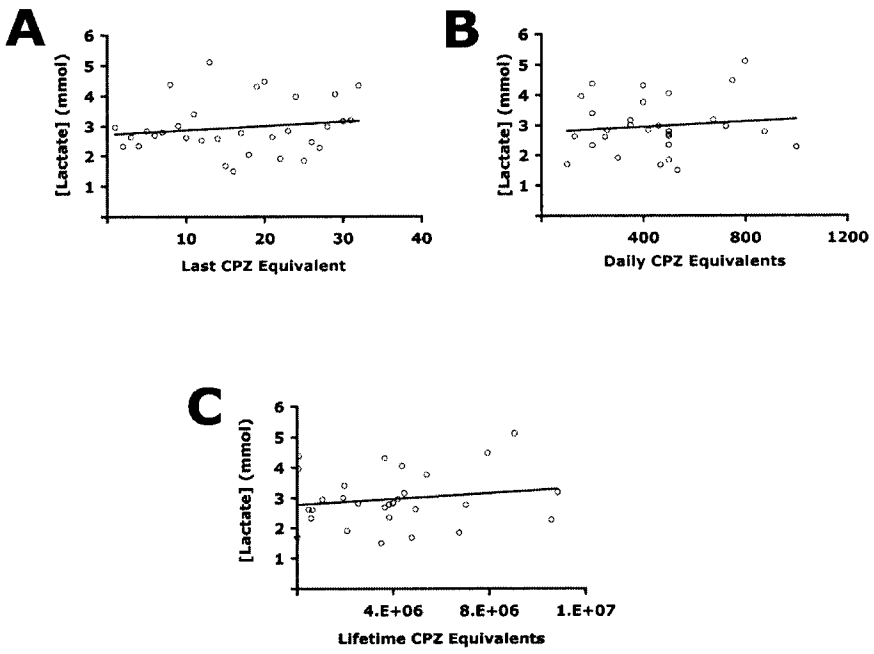

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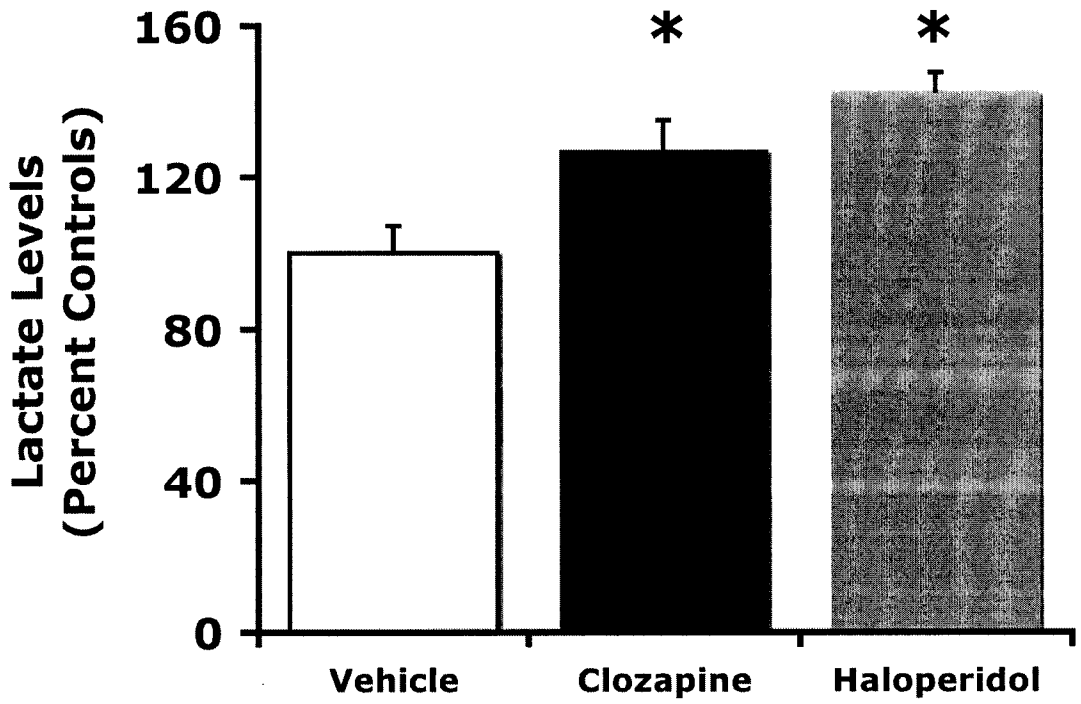
Figure 4



Chapter IV: General Discussion

Part I: The PDC Phosphorlyation as a Molecular Mechanism of Neuronal-Astrocytic Metabolic Exchange

This work describes the involvement of the PDC in the metabolic phenotype of astrocytes and neurons *in vitro* and suggests that PDC phosphorylation and inactivation may underlie the molecular mechanism of metabolic compartmentalization between astrocytes and neurons.

PDC Expression and Activity

We demonstrate that both astrocytes and neurons express the PDC but that astrocytes express higher levels of the PDC complex. We also demonstrate that the PDC is largely inactivated in astrocytes while in neurons its activity is near maximal. Consistent with inactivation of the PDC we observe that astrocytes release significant amounts of lactate.

Astrocytes are Highly Glycolytic

To maintain high glycolytic activity a cell must regenerate NAD^+. This is normally accomplished by the reduction of pyruvate to lactate via lactate dehydrogenase (LDH). Consistent with this notion we observe that LDH expression and activity is significantly greater in astrocytes than in neurons. Using live-cell NADH imaging we demonstrate a significantly higher rate of NADH production in cytosol of astrocytes than neurons reflecting the higher glycolytic activity of astrocytes.

PDC Regulating Kinases and Phosphatases

We also determined the expression of all known kinases and phosphatases of the PDC in both cell types. The results of this study demonstrate that all kinases (PDK1-4) and phosphatases (PDP1-2) are expressed in both cells types though we observed unique expression profiles. Consistent with the notion of tighter regulation of the PDC in astrocytes we observed that PDK2, PDK4, and PDP1 are expressed at higher levels in astrocytes than neurons. The expression of PDK1 was greater in neurons than in astrocytes while the expression of PDK3 and PDP2 was equal in both cell types.

Dichloroacetate Induced Dephosphorlyation of PDC

In an attempt to alter the metabolic phenotype of astrocytes we utilized dichloroacetate to induce the dephosphorlytaion of the PDC. We hypothesized that by reducing phosphorylation of the astrocytic PDC we would alter the metabolic phenotype of high levels of lactate release observed in astrocyte cultures. We demonstrate that DCA treatment in the doses utilized in this study are not toxic to astrocytes, yet significant toxicity is observed in neurons at all doses utilized. Nonetheless, in astrocytes DCA treatment was able to reduce phosphorylation at site 1 on E1α. This dephosphorylation resulted in a decrease in media lactate release.

Future Studies

The results of this study suggest that metabolic compartmentalization between astrocytes and neurons may result from the differential phosphorylation of the PDC. To further elucidate the involvement of the PDC in neuron-glia metabolic coupling it would

be useful to determine whether or not neurotransmitters or metabolic signaling molecules alter of the phosphorylation status of PDC in astrocytes or neurons. Along these lines it would be of interest to determine whether not an increase in calcium levels can alter the phosphorylation status and activity of the astrocytic PDC. Finally, we propose to determine whether or not metabolic coupling can be visualized via live-cell NADH imaging.

Part II: Lactate as a Biomarker of Schizophrenia

Lactate levels are Increased in the Cerebellum of Patients with Schizophrenia.

In an attempt to elucidate whether metabolic alterations, specifically an increase in postmortem lactate levels, are unique to the prefrontal cortex (PFC) we measured lactate levels in the cerebellum of patients with schizophrenia and control subjects. Unlike the prefrontal cortex, the cerebellum is a region of the brain not associated with schizophrenia. The prefrontal cortex regulates executive function and cognitive functioning; the cerebellum is involved with the coordination of movements. Thus increased lactate levels in the cerebellum would suggest that this phenomenon is not a primary feature of the disease. Indeed we demonstrate that lactate levels are significantly increased in the cerebellum of patients with schizophrenia. Thus we hypothesize that increased postmortem brain lactate levels are the result of antipsychotic drug administration inducing alterations in glucose metabolism that manifest in the postmortem period as lactate.

Lactate Levels are Highly Correlated with pH, Weakly Correlated with Age, and are not Correlated to Postmortem Interval.

We observe that the increase in lactate (or lactic acid) may account for some, though not all, of the decrease in postmortem brain pH observed in multiple brain collections. Decreased postmortem brain pH is commonly associated with tissue degradation or a high postmortem interval (PMI). We observe that pH is not correlated to PMI suggesting that the decrease in pH is not reflective of tissue degradation. We also observed a weak correlation between lactate levels and age at death. This finding may suggest that older patients have an increased agonal state that is accompanied by a prolonged semi-hypoxic period resulting in the incomplete metabolism of glucose. Alternatively this finding may result from an increase in astroglia numbers during aging. Glycogen stores are found almost exclusively in astrocytes in the CNS. During the very early postmortem periods glycogen stores are rapidly converted to glucose and glucose as well as other glycolytic metabolites are rapidly converted to lactate during the postmortem period.

Lactate levels do not Correlate to Any Measure of Chlorpromazine Equivalents.

We found no correlation of lactate levels with any measure (lifetime, daily, or last) of chlorpromazine (CPZ) equivalents. A common feature of all antipsychotics is the ability to antagonize dopamine signaling, specifically at the D_2 type receptor. Though typical antipsychotics are almost exclusively D_2 antagonists, atypical antipsychotics antagonize the D_2 as well as D_3 and D_4 dopamine receptors. Atypicals also regulate numerous other neurotransmitter systems such as the histamine (H_1) and serotonergenic

(5-HT_{2A} and 5-HT_{2C}) systems. All antipsychotics have a different pharmacological profile of antagonism at the D_2 receptors. CPZ equivalents are used as a relative index of D_2 receptor blockade and these values are determined by the drugs affinity for the receptor. Thus CPZ equivalents are used as relative index to compare the actions of different drugs. CPZ equivalents are extrapolated from patient's medical records hence these values are only used as an index of prescribed treatment and are not necessarily a true measure of an individual's treatment of drug history. Furthermore, CPZ equivalents do not account for the modulation of other receptor systems that may affect glucose metabolism.

Chronic administration of antipsychotic drugs to rodents results in an increase in postmortem lactate levels.

To directly test whether the increase in lactate levels observed in this study is the result of antipsychotic administration we treated rats with two different antipsychotics for 28 days and subsequently measured postmortem lactate levels. Rats were treated with either haloperidol the prototypical typical antipsychotic or clozapine, the prototypical atypical antipsychotic. Both haloperidol and clozapine administered at a physiological relevant dose increased postmortem brain lactate levels.

Future Studies

The results of this study suggest that increased levels of lactate in the postmortem brains of patients with schizophrenia are not a primary feature of the disease but are the result of antipsychotic treatment. Though antipsychotics treatment results in numerous

metabolic alterations, the exact mechanism(s) are unknown. Thus deciphering the molecular mechanism of this phenomenon would be of great interest. We would propose the following experiments. To determine whether or not treatment with antipsychotics result in increased glycogen levels *in vitro* and *in vivo*. Possible mechanisms for the increase in postmortem lactate levels are the conversion of glycogen stores to glucose and ultimately lactate during the postmortem period. To determine whether or not antipsychotic administration results in altered enzyme expression or activity levels, both *in vitro* or *in vivo*.